# Temperature sex determination, incubation duration, and hatchling sexual dimorphism in the Española Giant Tortoise (*Chelonoidis hoodensis*) of the Galápagos Islands

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*Abstract.*—Sex determination (SD) mode is documented in only 26% of turtle species; temperature dependent sex determination (TSD) is common but not ubiquitous. SD mode is documented for only five tortoise species; all of these have TSD with the Ia pattern. Temperature dependent sex determination was reported in Galápagos tortoises (*Chelonoidis nigra* complex) in 1991 based solely on a personal communication. Here we report TSD pattern, incubation duration, and hatchling sexual dimorphism in the Española Giant Tortoise (*Chelonoidis hoodensis*) of the Galápagos Islands based on experiments conducted in 1986–87. We found strong evidence for Type Ia TSD, a pivotal incubation temperature of 28.3 °C, and a range for transition temperatures of 25.2–31.4 °C. We also found longer incubation durations for male than for female hatchlings, and describe a new method for sex identification for hatchling tortoises. These results have important implications for incubation of eggs for head-starting captive breeding, especially for conservation purposes, and for interpretation of data from natural nests. We caution against the assumption that all *C. nigra* complex species have similar pivotal or transitional temperature ranges, and encourage evaluation of more species in this group.

*Resumen.*—El modo de determinación sexual (DS) solamente se ha documentado para el 26% de las especies de tortugas; la determinación del sexo por la temperatura (DST) en las tortugas es común pero no es generalizada. Se conoce el modo SD solamente para cinco especies de tortugas; todas ellas tienen el modo de DST. Se reportó en 1991 la determinación TSD para las tortugas de Galápagos (complejo *Chelonoidis nigra*), sobre la base de una comunicación personal. En este trabajo reportamos el patrón de DST, la duración de la incubación y el dimorfismo sexual a la eclosión en *Chelonoidis hoodensis* (la Tortuga Gigante de Española de las Islas Galápagos), sobre la base de experimentos realizados entre 1986–87. Nosotros encontramos firme evidencia para el DST tipo Ia, una temperatura pivotal de incubación de 28.3 °C y un rango de temperaturas transicionales de 25.2–31.4 °C. También detectamos que los períodos de incubación hasta la eclosión de tortugas machos fueron más prolongados en comparación con las hembras. Estos resultados tienen implicaciones ventajosas e importantes para la incubación de los huevos y para la interpretación de datos tomados de nidos naturales. Sugerimos evitar el inferir que todas las especies del complejo *C. nigra* tengan rangos de temperaturas transicionales similares y sugerimos la evaluación de más especies dentro de este grupo.

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## Introduction

Sex determination (SD) mode is documented in only 86 (26%) of the approximately 335 known turtle species; temperature dependent sex determination (TSD) is common but is not ubiquitous (The Tree of Sex Consortium 2014a, b). In the family Testudinidae (tortoises, ca. 57 extant species, TTWG 2017), SD mode is documented for only five species: *Testudo hermanni* (Eendebak 1995), *T. graeca* (Pieau 1971), *Gopherus agassizii* (Spotila et al. 1994), *G. polyphemus* (Burke et al. 1996; Demuth 2001), and *Malacochersus tornieri* (Ewert et al. 2004); all have TSD Type Ia. Two other Testudinidae ("*Geochelone elephantopus*" = *Chelonoidis nigra* complex and "*G. gigantea*" = *Aldabrachelys gigantea*) were reported as TSD in Janzen and Paukstis (1991), however both reports were based on unpublished data. The source data for *C. nigra* complex was unclear but presumably based on unpublished work by Sancho (1988) (Janzen, pers. comm.).

*Chelonoidis* is the largest tortoise genus (ca. 15 extant species, TTWG 2017); all *Chelonoidis* species are South American and most (10–12) *Chelonoidis* species are in the *C. nigra* complex (Galápagos giant tortoises) (van Dijk et al. 2014; Poulakakis et al. 2015; TTWG 2017). Populations of Galápagos giant tortoises have been greatly reduced, in some cases to extinction, due to predation by humans and by interactions with introduced species (MacFarland et. al. 1974a, b). Captive rearing of several *Chelonoidis* species for repatriation to their islands of origin has been an important part of Galápagos conservation programs (Cayot et al. 1994; Cayot 2008). These programs have become increasingly sophisticated, now including genetic analyses (Russello et al. 2010; Milinkovitch et al. 2013) and studies of the impact that repatriations have on vegetation (Gibbs et al. 2008).

The discovery that sex is determined by incubation temperature in most turtles has been of interest to the coordinators of Galápagos giant tortoise conservation programs for decades. This is because detailed knowledge of the effects of incubation temperature on hatchling sex could help managers avoid obvious pitfalls, such as producing all males, and to deliberately manipulate sex ratios (Vogt 1994). However, SD studies of *Chelonoidis* have not progressed because sexually dimorphic characteristics typically take many years to develop and it is unacceptable to conduct risky procedures on individuals so valuable to conservation. Therefore, the development of quick, easy, and harmless ways to identify the sex of hatchlings (e.g., Burke et al. 1994; Mrosovsky et al. 1999; Valenzuela et al. 2004; Martínez-Silvestre et al. 2015) are potentially very valuable.

Typically, investigations of TSD target four parameters: 1) the TSD pattern (Ewert and Nelson 1991), 2) the pivotal (=threshold; Bull et al. 1982) temperature, (= the constant incubation temperature that results in 1:1 offspring sex ratios, Mrosovsky and Pieau 1991), 3) the transitional range of incubation temperatures (TRT) (= the range of constant incubation temperatures that produce both sexes), and 4) the temperature-sensitive period (TSP) (= portion of the incubation period during which incubation temperature can affect hatchling sex, Bull and Vogt 1981). We sought to identify the SD mode, pivotal temperature, and TRT of the Española Giant Tortoise (*Chelonoidis hoodensis*) of the Galápagos Islands and develop ways to identify hatchling sex using external morphology and incubation duration. This species has been the subject of long term conservation efforts (Gibbs et al. 2014). Española Giant Tortoises were reduced to just 15 individuals by 1960; these were brought into captivity 1963–1974 and became the parents of thousands of offspring (Cayot et al. 1994; Cayot 2008; Márquez et al. 1991). Nearly 1,500 offspring have been released onto Española, and successful reproduction was first observed starting in 1990 (Márquez et al. 1991; Cayot et al. 1994; Cayot 2008). Although *C. hoodensis* remains Critically Endangered (CITES I, IUCN Red List), this is clearly an example of a highly successful chelonian head-starting program, despite low levels of genetic variation (Milinkovitch et al. 2013).

## Materials and Methods

### *Incubation of eggs at different temperatures*

A total of 189 *Chelonoidis hoodensis* eggs laid in 1986 were incubated at three temperatures: 25.5, 29.5, and 33.5 °C (67 eggs at each temperature) at the Galápagos Rearing Center, Puerto Ayora, Santa Cruz, Galápagos, Ecuador. Eggs were placed in plastic boxes with damp vermiculite; boxes were covered and placed in incubation chambers at constant temperatures. Boxes were rotated inside the incubators once per week to avoid effects of any thermal gradients in the chamber (Gutzke and Paukstis 1983). Incubation data were also collected from six additional tortoise hatchlings incubated and hatched earlier in the same facility.

### *Sex identification*

Hatchling sex was identified in three ways: by direct gross observation of gonads, histological examination of gonads, and by laparoscopy. The gonads from 35 young tortoises that died of natural causes were examined via both direct gross observations of gonads and histological examinations of gonads. In some cases, the gonads were removed and fixed soon after the tortoise's death. However, most samples came from tortoises that were preserved intact either in formalin or alcohol. The gonads were embedded in Paraplast, cut at 5 μm thickness and stained with Harris' Hematoxylin and Eosin yellow stains. The histological procedures are described in Sancho (1988). Samples from tortoises fixed in alcohol

produced very poor histological sections and the gonads could not be identified. Fixations in formalin was also poor, but the gonads could be identified (Sancho 1988).

Laparoscopies were performed on 15 additional young tortoises; using standard surgical techniques. A small incision was made in the inguinal pocket just anterior to tortoises' hind legs to permit examination of the gonads. After observation, the skin was sutured and bathed with an antiseptic solution. We also counted the number of large dorsal scales in the tails of these individuals.

We assessed SD mode and estimated both pivotal temperature and TRT using the program TSD 4.0.3 (Girondot 1999, 2012; Godfrey et al. 2003) as in Burke and Calichio (2014). This program uses a maximum likelihood approach with a rather simple mathematical equation to compare the fit of observed data to four different sex determination models (genotypic sex determination, TSD IA, IB, and II) and uses Akaike Information Criterion (AIC) to rank the different models by penalizing for more parameters. The minimum data requirement for the TSD 4.0.3 program is sex ratio data from at least two constant temperature incubation experiments in which both sexes are produced.

## Results

### The juvenile gonads of Chelonoidis hoodensis

We examined the tortoise gonads both macroscopically and histologically; there was complete agreement between sex identification according to the gross morphology and the histology of gonads (Sancho 1988). The characteristics of juvenile gonads in *C. hoodensis* were similar to those of other turtles (Gutzke and Bull 1986), they consisted of two parts, the cortex and the medulla. The testicles of the juvenile tortoises (of up to two years of age) were white cylindrical structures of 7 to 8 mm in length, located on the ventral surface of the kidney. Testicles had a uniform reticular pattern of vascularization and the cortex was thin. Males lacked Müellerian ducts (or oviducts). Ovaries in juvenile tortoises, in contrast, were longer, thicker and flatter than testicles (mean length 11 mm). Vascularization was restricted to the medulla and the cortex was thick. In females, sex identification was aided by the presence of Müellerian ducts.

Germ cells were found in the medulla of males and in the cortex of females (Sancho 1988). Germ cells were rounder and larger than the somatic cells of the gonads. In one individual, germ cells were found both in the cortex and the medulla; in this embryo sex was not yet determined.

### Effect of the temperature of incubation on sex determination

For unknown reasons, many embryos died during early incubation and others died during the last stages of incubation or at the time of hatching. Ten of the 11 hatchlings (91% male, hatch rate = 16.4%) from eggs incubated at 25.5 °C were identified as males, one was a female. At 29.5 °C, 27 (hatch rate = 40.3%) tortoises hatched and survived. We were able to identify sex in only 15 of these. Five of the 15 sexable hatchlings from eggs incubated at 29.5 °C were identified as males, 10 were female (33% male). All of the eggs incubated at 33.5 °C died during early development.

Results of the Hill and logistic models (program TSD 4.0.3) were indistinguishable using AIC (both AIC values = 8.99, Akaike weights = 0.50, goodness of fit < 0.001). This is strong evidence for Type Ia TSD. The logistic model predicted a pivotal incubation temperature of 28.3 °C (S.E. = 0.24), and a range for transition temperatures (TRT) of 25.2 °C (S.E. = 0.56)–31.4 °C (S.E. = 0.55). The Hill model predicted a pivotal incubation temperature of 28.3 °C (S.E. = 0.25), and a range for transition temperatures (TRT) of 25.2 °C (S.E. = 0.24)–31.5 °C (S.E. = 0.29).

Incubation duration for male hatchlings ranged from 125–167 days ($\bar{x}$ = 141.7) and incubation duration for female hatchlings ranged from 111–122 (x = 118.9). Incubation duration for males was significantly longer than for females ($t$ = 4.24, d.f. = 18, two tailed $P$ < 0.001).

The number of large dorsal scales in the tails of hatchlings identified as males ranged from 4–7 ($n$ = 10, $\bar{x}$ = 4.9), females ranged from 2–5 ($n$ = 10, $\bar{x}$ = 3.7). Male hatchlings had significantly more large dorsal scales on their tails than did females ($t$ = 2.48, d.f. = 18, two tailed $P$ = 0.023).

## Discussion

Our finding that the Española Giant Tortoise (*Chelonoidis hoodensis*) has TSD is not surprising because this was reported by Sancho (1988) and is well known by the managers in charge of the Galápagos Tortoise Captive Breeding Program (Marquez et al. 1999; Burke, pers. obs.). However, we have added considerable detail to previously vague reports, including the pivotal temperature and the range for transition temperatures. These findings can inform captive breeding programs and field studies. For example, this type of information has been used in other species to predict hatchling sex ratios in natural nests (Georges et al. 1994; Delmas et al. 2008; Grosse et al. 2014).

Our finding that eggs incubated at female-producing temperatures and eggs incubated at male-producing temperatures differed in incubation duration is also not surprising, because the negative correlation between incubation temperature and incubation duration is well known for many turtles (e.g., Yntema 1978; Mrosovsky and Yntema 1980; Booth 1998). However, although this knowledge is commonly used in studies of sea turtles (e.g., Mrosovsky et al. 1999) to predict sex ratios of natu-

ral nests, we could find no similar studies in other turtles. We suggest that incubation duration could be used more commonly to predict sex in both artificially incubated eggs and eggs incubated *in situ.*

We consider our results indicating that female *C. hoodensis* had fewer large scales on the dorsal aspects of their tails interesting but needing additional investigation, especially a standardization of the method of counting tail scales. If the number of tail scales is sexually dimorphic, this technique could provide an extremely convenient way to sex hatchlings, and could be potentially valuable to many studies. We point out that incubation temperature is known to affect many hatchling characteristics, such as survivorship, body size, locomotor performance, and growth (e.g., Janzen 1993; Roosenburg and Kelley 1996; Demuth 2001). In addition, Burke et al. (1994), Valenzuela et al. (2004), and Lubiana and Júnior (2009) found significant sexual dimorphisms in body size and shape in hatchling turtles, while tail length is commonly sexually dimorphic in turtles as well (e.g., Casale et al. 2005).

Our results on pivotal temperature, transitional temperatures, and incubation duration should not be assumed to be identical in other *Chelonoidis*, even other *C. nigra* complex species. Variation in TSD patterns can occur between closely related turtle species (Bull et al. 1982; Ewert et al. 1994; Ewert et al. 2004) and even within a species (Ewert et al. 2005).Because of the diverse nesting microhabitats used by *C. nigra* complex species (Burke, pers. obs.), there may be considerable diversity in pivotal temperatures, TRT, and TSP.

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**Ana Sancho** (1965–2009) was an Ecuadorian biologist with an MBA specialized in project management, dedicated her work to the conservation of biodiversity, particularly in the Galápagos Islands. One of her research projects showed the link between Galápagos giant tortoises' sex and their eggs' incubation temperature. Later on, as Fishing Officer for South America at the NGO Traffic, she researched and coordinated the publication of the Report of Fishery activities and Trade of Patagonian Toothfish, which was presented at the Commission for the Conservation of Antarctic Marine Living Resources; as well as the Report on Sea Cucumber Trade in the Galapagos Islands. Between 2004 and 2008, she worked as coordinator of the UNDP/GEF project for the Control of Invasive Species in the Galápagos Archipelago. Among her main achievements was the establishment of a trust fund to control invasive species of the archipelago, which raised over $15 million. Her last professional activity was as coordinator of the project for the Implementation of Early Warning Systems and Natural Risk Management in 2009. She published several books and was part of Ecuador's official delegations in conservation events around the world. Apart from her extraordinary professional legacy, her friends and family remember her for her love and determination.



**William H. N. Gutzke** was a well-known herpetologist who studied embryonic development and phenotypic plasticity of reptiles and amphibians at both Memphis State University and the University of Memphis. Bill completed his Ph.D. (1984) on the influence of environmental factors on eggs and hatchlings of painted turtles (*Chrysemys picta*) and did post-doctoral work with James Bull at the University of Texas. He subsequently published 30+ articles in scientific journals, mentored four Ph.D. students, two Master's students, and at least 60 undergraduates. Bill Gutzke passed away in 2004.

**Howard L. Snell** is a professor in the Biology Department of the University of New Mexico and Curator of the Herpetology Division of the Museum of Southwestern Biology, also at UNM. Howard and his wife Heidi started work in the Galápagos Islands as volunteers from the US Peace Corps at the Charles Darwin Research Station in 1977. They continued visiting the archipelago through 2004. Within that interval they were variously based at Colorado State University, Texas Christian University, and Memphis State University before settling at the University of New Mexico in 1986. Howard worked with the Charles Darwin Foundation / Research Station as Program Leader for Reptiles, Vice President for North America, Program Leader for Vertebrate Ecology & Monitoring, and Director of Science Programs.



**Solanda Rea** became part of the Charles Darwin Research Station in 1983 when she started working as Herpetology Assistant with the Giant Tortoise Breeding Program. She currently works with the Visiting Scientists Program and has a key role managing the sample exportation process. In addition, Solanda has been in charge of the meteorological station since 1994, ensuring the collection and registration of data which is an important tool in the analysis of environmental events that influence the Galápagos Islands.



**Marcia Wilson** is the program manager for the National Park Service (NPS) Chihuahuan Desert Inventory and Monitoring (I&M) Network. She has been working with the NPS I&M program since 2003. Prior to her time with NPS, she was Deputy Chief for the Branch of Migratory Birds Research at Patuxent Wildlife Research Center (PWRC) where she conducted research on wintering migratory birds in southern Mexico. Her first position with PWRC was as Leader of the Puerto Rico Research Group. She was responsible for the captive-breeding program and the wild flock management of the endangered Puerto Rican Parrot. She began her career as Head of the Charles Darwin Terrestrial Ecology Department located on the Galápagos Islands of Ecuador.



**Russell L. Burke** is the Donald E. Axinn Distinguished Professor of Ecology at Hofstra University in New York. He has been conducting research on reptiles for over 30 years, mostly focusing on the ecology and conservation of turtles. He has published 50+ scientific articles, numerous publications for the general public, and mentored 28 Master's students. Each year he runs a large citizen science project exploring the ecology of Diamondback Terrapins in Jamaica Bay, New York, and he regularly takes groups of college students to the Galápagos islands for field ecology classes.